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Antimalarial Activity of New Water-Soluble Dihydroartemisinin Derivatives[1]

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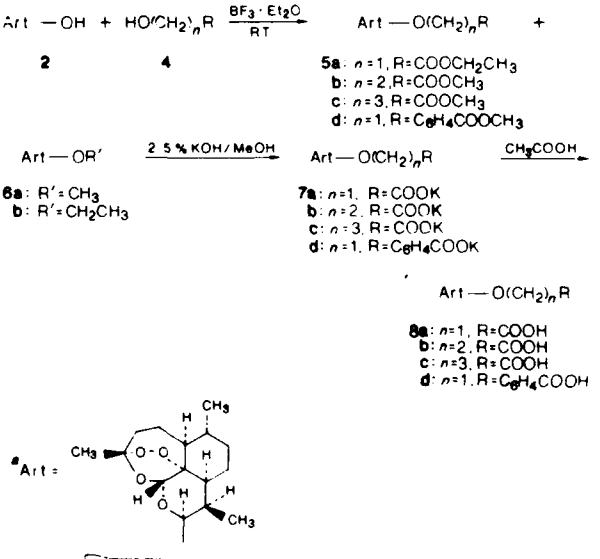
The usefulness of sodium artesunate (**3**), a water-soluble derivative of artemisinin (**1**), is impaired by its poor stability in aqueous solution. To overcome the ease of hydrolysis of the ester group in **3**, a new series of derivatives of dihydroartemisinin (**2**) was prepared in which the solubilizing moiety, which contains a carboxylate group, is joined to dihydroartemisinin by an ether rather than an ester linkage. The new derivatives were prepared in good yield by treatment of dihydroartemisinin with an appropriate alcohol under boron trifluoride etherate catalysis at room temperature. All major condensation products are the β isomer. Hydrolysis of the esters with 2.5% KOH/MeOH gave the corresponding potassium salts, which were converted to free acids (**8b–d**) by acidification. The derivatives were tested in vitro against two clones of human malaria, *Plasmodium falciparum* D-6 (Sierra Leone clone) and W-2 (Indochina clone). No cross-resistance to the antimalarial agents mefloquine, chloroquine, pyrimethamine, sulfadoxine, and quinine was observed. In general, the new compounds are more effective against the W-2 than the D-6 strain. Esters (**5a–d**) possess activity comparable to that of the parent compounds **1** and **2**; however, conversion of the esters to their corresponding carboxylates (**7a–d**) or acids (**8b–d**), with the exception of artelinic acid (**8d**), drastically decreases the antimalarial activities in both cell lines. Artelinic acid, which is both soluble and stable in 2.5% K_2CO_3 solution, possesses superior in vivo activity against *Plasmodium berghei* than artemisinin or artesunic acid.

Artemisinin[1] (*qinghaosu*, **1**), a clinically useful antimalarial agent that was isolated from the plant *Artemisia annua*, is an unusual sesquiterpene lactone containing an epidioxide function.[2-8] Dihydroartemisinin (**2**), obtained by sodium borohydride reduction of **1**, was reported[9] to be more therapeutically active than the parent compound.

Scheme I[a]



Neither **1** nor **2** exhibit cross-resistance to chloroquine and both were proven efficacious against cerebral malaria in humans.[6] Sodium artesunate (**3**), the salt of the succinic acid half-ester derivative of dihydroartemisinin, is water soluble and can be administered by intravenous injection. This makes the compound particularly useful in the

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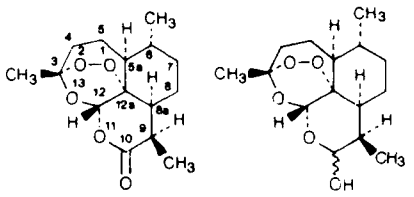

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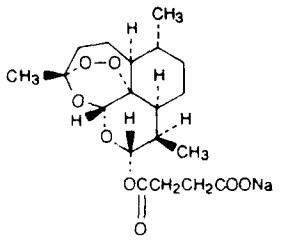

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treatment of cerebral malaria where rapid reversal of the parasitemia and restoration to consciousness of the patient is critical.[6] The utility of sodium artesunate is, however, impaired by its poor stability in aqueous solution due to the ease of hydrolysis of the ester linkage.

In order to overcome the stability problem, we have prepared a series of new water-soluble, stable derivatives in which the solubilizing group, carboxylate, is on a moiety that is joined to dihydroartemisinin by an ether rather than an ester linkage.

Chemistry

Dihydroartemisinin (**2**) was prepared by sodium borohydride reduction of **1** according to a modified literature[2] procedure. The peroxide group is not affected by the sodium borohydride treatment. Inasmuch as dihydroartemisinin is a hemiacetal (lactol), it exists as a mixture of α and β anomers whose ratio is solvent dependent.[10]

The new ether derivatives of dihydroartemisinin (**5a–d**) were prepared by treatment of **2** with an appropriate alcohol (**4**) under the catalysis of boron trifluoride etherate at room temperature (Scheme I). The yield of the purified condensation products (**5a–d**) ranged from 70% to 90%. A minor product, **6a** or **6b**, depending on whether the ester **4** is the methyl or ethyl ester, was also isolated in some of the reactions. The methanol or ethanol needed for the formation of **6a** or **6b** is very likely generated by transesterification or lactone formation in **4**.

All major condensation products **5** are the β isomer as indicated by the small coupling constants between 10-H (J = 3–4 Hz) and 9-H.[11] Due to the close proximity to several asymmetric carbon centers on the dihydroartemisinin moiety, the two methylene protons on the carbon α to the new ether oxygen are nonequivalent and thus appear as an AB quartet. A large geminal coupling constant (J = 12.6 Hz) and chemical shift difference ($\Delta\delta$ = 0.4 ppm) between the two benzylic protons of **5d** was observed. Likewise, a large coupling constant between the methylene protons α to the ether oxygen of **5b** and **5c** can be measured by a decoupling technique. A similar observation has been disclosed in the literature.[11] However, the corresponding methylene protons of **5a** appear as a singlet, a surprising exception.

The hydrolysis of ester **5a** with 2.5% KOH/MeOH gave the corresponding potassium salt **7a**, which was purified by reverse-phase chromatography. Sodium salts could be prepared in an identical manner. Conversion of the salt (**7a**) to the free acid (**8a**) was achieved by acidification with acetic acid. The free acid appears to be unstable, as evidenced by gradual changes seen in its ^{1}H NMR spectrum on standing. This may be due to the instability of the acetal or ketal functions of the molecule under acidic conditions. In contrast, **7b–d** were smoothly converted to their corresponding stable free acids **8b–d**.

To compare the aqueous stability of the new derivatives with artesunic acid, the rates of hydrolysis of **8d** and **3** were studied in 2.5% K_2CO_3/D_2O solution at room temperature and monitored by ^{1}H NMR. In a similar manner, the stability of artesunic acid was examined in aqueous $NaHCO_3$.

Table I. In Vitro Antimalarial Activities against *P. falciparum*

	IC_{50}, ng/mL			IC_{50}, ng/mL	
no.	Sierra Leone (D-6)	Indochina (W-2)	no.	Sierra Leone (D-6)	Indochina (W-2)
1	2.93	0.66	**7b**	85.02	23.28
2	0.41	0.69	**7c**	75.52	10.52
5a	0.60	0.26	**7d**	1.74	0.92
5b	1.84	0.64	**8b**	51.74	35.64
5c	3.06	0.95	**8c**	17.90	8.04
5d	0.77	0.37	**8d**	4.07	1.38
7a	53.79	26.23			

Table II. Antimalarial Activity of Artelinic Acid (**8d**) and Related Compounds against *P. berghei* in Mice

compound	dosage, mg/kg	vehicle	no. of cures
artelinic acid (**8d**)	640	5% $NaHCO_3$	5/5
	160	5% $NaHCO_3$	5/5
	40	5% $NaHCO_3$	5/5
artemisinin (**1**)	640	peanut oil	5/5
	160	peanut oil	5/5
	40	peanut oil	3/5 (A),[a] 2/5
artesunic acid (**3**)	640	5% $NaHCO_3$	5/5
	160	5% $NaHCO_3$	3/5 (A), 0/5
	40	5% $NaHCO_3$	2/5 (A), 0/5

[a] A = active. The terms cure and active are defined in the Experimental Section.

Results and Discussion

The new water-soluble dihydroartemisinin derivatives were tested in vitro against two clones of human malaria, *Plasmodium falciparum* D-6 (Sierra Leone clone) and W-2 (Indochina clone). The former clone is a strain that is resistant to mefloquine and the latter to chloroquine, pyrimethamine, sulfadoxine, and quinine.

The results (Table I) indicate that the new derivatives, like the parent agents **1** and **2**, are not cross-resistant to any of the antimalarial agents mentioned above. The derivatives are, in general, more effective against the W-2 than the D-6 strain. Esters (**5a–d**) possess activity comparable to that of the parent compounds, **1** and **2**, although activity decreases as the aliphatic chain is elongated from one (**5a**) to three carbons (**5c**). Conversion of the esters (**5a–d**) to their corresponding carboxylates (**7a–c**) or acids (**8b–d**), with the exception of **7d** and **8d** (artelinic acid), drastically decreases the antimalarial activities in both cell lines.

Overall, the free acids exhibit better in vitro antimalarial activities than their salts; however, **7d**, the salt form of **8d**, is not only water soluble and stable in solution but also possesses comparable in vitro activity to artemisinin (**1**). Because artelinic acid appears to be the most promising of the series, further studies on its in vivo antimalarial activity were assessed in a *Plasmodium berghei* mouse

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Table III. Stability of Artesunic (3) and Artelinic (8d) Acids in 2.5% K_2CO_3/D_2O Solution

time	% hydrolysis 3	% hydrolysis 8d	time	% hydrolysis 3	% hydrolysis 8d
1.5 h	20	0	96 h	98	0
24 h	60	0	35 days	100	0
48 h	75	0	67 days	100	<3
72 h	91	0			

Table IV. Stability of Artesunic Acid (3) in 5% $NaHCO_3/D_2O$ at Room Temperature

time	% hydrolysis	time	% hydrolysis
4 h	<5	4 days	45
2 days	22	8 days	75
3 days	33	9 days	82

screen side-by-side with the reference compounds, artemisinin (1) and artesunic acid (3). The results are shown in Table II.

At a high dose level (640 mg/kg), all three compounds, 1, 3, and 8d, showed complete cures in the mice treated (5/5). At a lower dose level (160 mg/kg), artesunic acid was active, but there were no 60-day survivors among the treated mice. Artemisinin and artelinic acid showed 5/5 cures at the same dose level. However, at the lowest dose level of 40 mg/kg, artelinic acid still showed a 100% cure, whereas artemisinin showed 3/5 active and 2/5 cure. In contrast, none of the mice treated with artesunic acid showed 60-day survival. The results indicate that the new analogue **8d** possesses superior in vivo activity against *P. berghei* than artemisinin or artesunic acid.

The stability studies performed in 2.5% K_2CO_3/D_2O revealed that no detectable changes occur in **8d** after 35 days and <3% hydrolyzed after 67 days at room temperature, whereas substantial hydrolysis (20%) took place in **3** under identical conditions within 1.5 h, with nearly complete hydrolysis taking place after 4 days (Table III). The rate of hydrolysis of **3** in 5% $NaHCO_3/D_2O$ solution was found to be slower than in 2.5% K_2CO_3/D_2O. The half-life was estimated to be about 4.5 days in 5% $NaHCO_3$ (Table IV) compared to <1 day in 2.5% K_2CO_3 solution.

The superior in vivo antimalarial activity and stability in aqueous solution of **8d** to artesunic acid renders the new compound a potential candidate drug for the treatment of cerebral malaria.

Experimental Section

Chemistry. All melting points were determined on a Thomas-Hoover melting point apparatus and are uncorrected. Infrared spectra of solid samples were obtained in KBr disks on a Perkin-Elmer Model 283 spectrophotometer. NMR spectra were run on a JEOL FX90Q spectrometer using Me_4Si as an internal standard. Analyses were performed by Spang Microanalytical Laboratory, Eagle Harbor, MI.

Dihydroartemisinin (2). Artemisinin (1; 0.5 g, 1.8 mmol)[7] in 40 mL of MeOH was cooled in an ice bath to 0–5 °C. To the solution was added in small portions 0.25 g (6.6 mmol) of $NaBH_4$ over a period of 30 min. The solution was stirred at 0–5 °C for 2 h after the addition of $NaBH_4$ was complete, and the solution was neutralized with 30% AcOH/MeOH and evaporated to dryness under reduced pressure. The white residue was extracted three times with 50 mL of EtOAc. The EtOAc extracts were combined, filtered, and evaporated to dryness to give 0.38 g (75%) of white needles, mp 152–154 °C. Recrystallization from EtOAc/hexane raised the melting point to 153–155 °C (lit.[2] mp 153–154 °C).

Ethyl 2-(10-Dihydroartemisininoxy)acetate (5a). Dihydroartemisinin (2; 0.5 g, 1.75 mmol) was dissolved in 70 mL of anhydrous Et_2O. To the solution were added successively 0.5 g (5 mmol) of ethyl glycolate and 0.25 mL of $BF_3{\cdot}Et_2O$. The reaction mixture was stirred at room temperature for 24 h, washed successively with 5% aqueous $NaHCO_3$ and H_2O, dried over Na_2SO_4, and evaporated to dryness under reduced pressure. The resultant oil was purified by preparative TLC using EtOAc/hexane (1:3, v/v) to give 0.45 g (68%) of **5a**: mp 50–52 °C; IR (neat) 1755 cm^{-1} [OC(=O)]; ^1H NMR ($CDCl_3$) δ 5.53 (s, 1 H), 4.87 (d, J = 3.6 Hz, 1 H), 4.26 (s, 2 H), 4.18 (q, J = 7.2 Hz, 2 H), 2.68 (m, 1 H), 1.43 (s, 3 H), 1.27 (t, J = 7.2 Hz, 3 H), 1.01 (d, J = 4.5 Hz, 3 H), and 0.93 (d, J = 1.8 Hz, 3 H). Anal. Calcd for $C_{19}H_{30}O_5$: C, 61.62; H, 8.11. Found: C, 62.09; H, 8.09.

The minor product (22% yield), with a higher R_f value than **5a**, was identified by NMR as the ethyl ether of **2** (**6b**, arteether):[11] ^1H NMR ($CDCl_3$) δ 0.90 (d, J = 7.2 Hz, 3 H), 0.96 (d, J = 3.6 Hz, 3 H), 1.18 (t, J = 7.2 Hz, 3 H), 1.43 (s, 3 H), 2.61 (m, 1 H), 3.47 (m, 1 H), 3.85 (m, 1 H), 4.84 (d, J = 3.6 Hz, 1 H), and 5.40 (s, 1 H); ^{13}C NMR ($CDCl_3$) ppm 12.86, 15.08, 20.22, 24.34, 24.61, 26.68, 30.74, 34.58, 36.37, 37.35, 44.44, 52.51, 63.62, 81.01, 87.73, 101.54, and 103.88.

Methyl 3-(10-Dihydroartemisininoxy)propionate (5b). The procedure for the preparation of **5a** was used to prepare **5b** by treating 0.5 g (1.75 mmol) of **2** with 1 g (9.6 mmol) of methyl 3-hydroxypropionate[12] (1 g, 9.6 mmol). It yielded 460 mg (70%) of the desired product as an oil after purification by preparative TLC (silica gel, EtOAc/hexanes, 1:2, v/v). The compound solidified on standing: mp 76–78 °C; IR (neat) 1743 cm^{-1} [OC(=O)]; ^1H NMR ($CDCl_3$) δ 5.43 (s, 1 H), 4.80 (d, J = 3.3 Hz, 1 H), 4.10 (m, 1 H), 3.68 (s, 3 H), 3.67 (m, 1 H), 2.58 (t, 2 H), 1.44 (s, 3 H), 0.95 (d, J = 6.3 Hz, 3 H), and 0.87 (d, J = 7.2 Hz, 3 H). Anal. Calcd for $C_{19}H_{30}O_7$: C, 61.62; H, 8.11. Found: C, 62.03; H, 8.07.

Methyl 4-(10-Dihydroartemisininoxy)butyrate (5c). The same procedure for the preparation of **5a** was used to prepare **5c**. Treatment of 1 g (8.5 mmol) of methyl 4-hydroxybutyrate[13] with 0.5 g (1.75 mmol) of **2** gave 70% of the desired product as an oil after purification: IR (neat) 1740 cm^{-1} [OC(=O)]; ^1H NMR ($CDCl_3$) δ 5.38 (s, 1 H), 4.77 (d, J = 3.6 Hz, 1 H), 3.85 (m, 1 H), 3.68 (s, 3 H), 3.34 (m, 1 H), 1.43 (s, 3 H), 0.96 (d, J = 3.6 Hz, 3 H), and 0.90 (d, J = 7.2 Hz, 3 H). Anal. Calcd for $C_{20}H_{32}O_7$: C, 62.50; H, 8.33. Found: C, 63.13; H, 8.64.

The minor product, with a higher R_f value than **5c** (22% yield), was identified by NMR as the methyl ether of **2** (**6a**, artemether):[11] ^1H NMR ($CDCl_3$) δ 0.90 (d, J = 6.3 Hz, 3 H), 0.96 (d, J = 2.7 Hz, 3 H), 1.44 (s, 3 H), 2.6 (m, 1 H), 3.42 (s, 3 H), 4.68 (d, J = 3.6 Hz, 1 H), and 5.38 (s, 1 H); ^{13}C NMR ($CDCl_3$) ppm 12.95, 20.37, 24.49, 24.70, 26.22, 30.93, 34.67, 36.46, 37.43, 44.53, 52.60, 55.60, 81.10, 87.76, 103.36, and 104.07.

Methyl *p*-[(10-Dihydroartemisininoxy)methyl]benzoate (5d). Treatment of 1 g (6.0 mmol) of methyl *p*-(hydroxymethyl)benzoate and 0.5 g (1.75 mmol) of **2** as given for the preparation of **5a** gave **5d** in 89% yield as an oil: IR (neat) 1725 cm^{-1} [OC(=O)]; ^1H NMR ($CDCl_3$) δ 8.02 (d, J = 9.0 Hz, 2 H), 7.38 (d, J = 9.0 Hz, 2 H), 5.45 (s, 1 H), 5.00 (d, J = 12.6 Hz, 1 H), 4.92 (d, J = 3.6 Hz, 1 H), 4.57 (d, J = 12.6 Hz, 1 H), 3.91 (s, 3 H), 1.45 (s, 3 H), 0.97 (d, J = 7.2 Hz, 3 H), and 0.95 (d, J = 3.6 Hz, 3 H). Anal. ($C_{24}H_{32}O_7$) C, H.

Potassium 2-(10-Dihydroartemisininoxy)acetate (7a). Ester **5a** (0.24 g, 0.65 mmol) was dissolved in 10 mL of 2.5% KOH/MeOH solution and allowed to stand at room temperature for 2 days. The solvent was reduced to half volume and diluted with an equal volume of H_2O. The solution was passed through a reverse-phase column [EM Reagents, Lobar prepacked column size B (310-25), Lichroprep RP-8, 40–63 μm] and eluted with MeOH + H_2O (1:1, v/v). The fractions were monitored by reverse-phase TLC (MeOH + H_2O, 1:1, v/v), and those which contained the desired compound were pooled, and the MeOH was evaporated under the reduced pressure. The aqueous solution was lyophilized to give 0.15 g (62%) of white crystals of **7a**, mp 157–159 °C. Anal. ($C_{17}H_{25}O_7K{\cdot}1.5\ H_2O$) C, H.

Prepared by the same procedure were the following compounds.

Potassium 3-(10-Dihydroartemisininoxy)propionate (7b; 78%, mp 153 °C dec). Anal. ($C_{18}H_{27}O_7K{\cdot}H_2O$) C, H.

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(13) Bundgaard, H.; Larsen, C. *Int. J. Pharm.* **1980**, *7*, 169.

Potassium 4-(10-Dihydroartemisininoxy)butyrate (7c; 40%, mp 142 °C dec). Anal. ($C_{19}H_{29}O_7K\cdot H_2O$) C, H.

Potassium *p*-[(10-Dihydroartemisininoxy)methyl]-benzoate (7d; 60%, mp 158 °C dec). Anal. ($C_{34}H_{29}O_7K\cdot H_2O$) C, H.

3-(10-Dihydroartemisininoxy)propionic Acid (8b). Ester **5b** (0.4 g, 1 mmol) in 10 mL of 2.5% KOH/MeOH solution was allowed to stand at room temperature for 2 days. The solvent was then evaporated to dryness under the reduced pressure. The residue was dissolved in 10 mL of H_2O and the solution was washed two times with an equal volume of Et_2O. The aqueous layer was acidified with AcOH and the mixture was extracted two times with Et_2O. The Et_2O extracts were combined, dried over Na_2SO_4, and evaporated to dryness. The oily product crystallized from hexane/Et_2O to give white crystals (0.25 g, 70%): mp 160–162 °C; IR (KBr) 1740 cm^{-1} (COOH); ^1H NMR ($CDCl_3$) δ 0.88 (d, J = 7.2 Hz, 3 H), 0.94 (d, J = 3.6 Hz, 3 H), 1.44 (s, 3 H), 2.62 (t, J = 6.3 Hz, 2 H), 3.63 (m, 1 H), 4.14 (m, 1 H), 4.82 (d, J = 3.6 Hz, 1 H), 5.45 (s, 1 H), and 7.98 (br s, 1 H). Anal. ($C_{18}H_{28}O_7$) C, H.

4-(10-Dihydroartemisininoxy)butyric Acid (8c). The same procedure for the preparation of **8b** was used to prepare **8c** from **5c** in 60% yield. The product is an oil: ^1H NMR ($CDCl_3$) δ 0.90 (d, J = 7.2 Hz, 3 H), 0.96 (d, J = 3.6 Hz, 3 H), 1.43 (s, 3 H), 3.39 (m, 1 H), 3.90 (m, 1 H), 4.79 (d, J = 3.6 Hz, 1 H), and 5.39 (s, 1 H). Anal. ($C_{19}H_{30}O_7\cdot{}^1/_4H_2O$) C, H.

***p*-[(10-Dihydroartemisininoxy)methyl]benzoic Acid (8d, Artelinic Acid).** The same procedure for the preparation of **8b** was adapted to the preparation of compound **8d** from **5d** in 55% yield. Purification was achieved by recrystallization from MeOH/H_2O: mp 142–145 °C; IR (KBr) 1700 cm^{-1} (COOH); ^1H NMR ($CDCl_3$) δ 0.96 (d, J = 2.7 Hz, 3 H), 0.98 (d, J = 7.2 Hz, 3 H), 1.46 (s, 3 H), 2.71 (m, 1 H), 4.60 (d, J = 13.5 Hz, 1 H), 4.94 (d, J = 2.7 Hz, 1 H), 5.00 (d, J = 13.5 Hz, 1 H), 5.46 (s, 1 H), 7.42 (d, J = 8.1 Hz, 2 H), and 8.10 (d, J = 8.1 Hz, 2 H). Anal. ($C_{23}H_{30}O_7\cdot{}^1/_2H_2O$) C, H.

Stability Studies of Artelinic (8d) and Artesunic (3) Acids. To a 5-mm NMR tube containing 10 mg of sample was added 0.5 mL of 2.5% K_2CO_3/D_2O solution. The initial spectrum of the solution was taken within 5 min and again at intervals thereafter (cf. Table III). The extent of hydrolysis was estimated by the following equation:

$$\% \text{ decomposition} = (B/2A + B) \times 100$$

where A = integration of H-10 (5.80 ppm, d, J = 10.8 Hz, 1 H) and H-12 (5.71 ppm, s, 1 H) of artesunate and B = integration of the singlet at 2.42 ppm for the potassium succinate formed. With time, the intensity of potassium succinate (2.42 ppm) signal increases, while that of H-10 and H-12 of artesunate decreases. The stability study of **3** in 5% $NaHCO_3/D_2O$ was carried out by the same procedure.

Biology. (a) In Vitro Antimalarial Studies. The in vitro assays were conducted by using a modification of the semiautomated microdilution technique of Desjardins et al.[14] and Milhous et al.[15] Two *P. falciparum* malaria parasite clones, designated as Indochina (W-2) and Sierra Leone (D-6), were utilized in susceptibility testing. They were derived by direct visualization and micromanipulation[16] from patient isolates obtained by the Centers for Disease Control, Atlanta, GA in 1980 and 1982, respectively. The patients had acquired infections either in Vietnam or in Sierra Leone. The Indochina clone is resistant to the antimalarials chloroquine, sulfadoxine, pyrimethamine, and quinine, whereas the Sierra Leone is resistant to mefloquine but susceptible to chloroquine, quinine, sulfadoxine, and pyrimethamine. Test compounds were initially dissolved in DMSO and 70% ethanol and diluted in RPMI 1640 culture medium with 10% human plasma to 400-fold. Drugs were subsequently further diluted by using the Cetus Pro/Pette (Perkin-Elmer Corp., Norwalk, CT) over a range of (1.56–100) $\times 10^{-9}$ M. Parasite inocula (at 0.5% parasitemia and a 1% hematocrit) were incubated for 24 h and added to equimolar concentrations of each test compound prior to the addition of [^{3}H]hypoxanthine. After a further incubation of 18 h, particulate matter was harvested from each microtiter well by using an automated cell harvester (Skatron, Inc., Sterling, VA). Uptake of [^{3}H]hypoxanthine was measured by using a scintillation spectrophotometer (Model LS3801, Beckman Instruments, Irvine, CA). Concentration–response data were analyzed by nonlinear regression and the IC_{50} values (50% inhibitory concentrations) for each compound were calculated.

(b) In Vivo Antimalarial Studies. The suppressive blood schizonticidal and curative activities of artemisinin, artelinic acid, and artesunic acid were measured in a test where mice were infected with 5.98×10^5 *P. berghei* parasitized cells intraperitoneally on day 0. Test compounds were dissolved in either peanut oil or 5% $NaHCO_3$ aqueous solution and were administered subcutaneously once a day for 3 consecutive days commencing on day 3. The dose levels of compounds given were 640, 160, and 40 mg/kg per day. Blood films were taken on days 6, 13, and 20. Blood schizonticidal activity was determined by monitoring blood films for the appearance of parasites and for extended survival times compared to infected untreated controls. Mice surviving 60 days were considered cured. The infected untreated control mice (negative controls) died on either day 6 or 7. Compounds was considered active when the survival time of the treated mice was greater than twice the control mice, i.e., 12–14 days.

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